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A New Regulatory Element Modulates Homoserine Lactone-Mediated Autoinduction of Ti Plasmid Conjugal Transfer

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Conjugal transfer of the *Agrobacterium tumefaciens* nopaline-type Ti plasmid pTiC58 is induced by agrocinopines A and B, opines secreted by crown gall tumors induced by the bacterium. This regulation functions through the transcriptional repressor, AccR. However, actual transcription of the *tra* genes is regulated by autoinduction through the activator TraR and the substituted homoserine lactone second messenger, *Agrobacterium* autoinducer (AAI). We have identified a new regulatory element that modulates the response of TraR to AAI. The gene, called *traM*, suppresses TraR-AAI activation of transcription of *tra* genes carried on recombinant clones. The suppression could be relieved by increasing the expression of TraR but not by increasing AAI levels. *traM* is located between *traR* and *traAF* on pTiC58 and is transcribed in the clockwise direction. The 306-bp gene encodes an 11.2-kDa protein showing no significant relatedness to other proteins in the databases. Mutations in *traM* in pTiC58 conferred a transfer-constitutive phenotype, and strains harboring the Ti plasmid produced easily detectable amounts of AAI. These same mutations engineered into the transfer-constitutive Ti plasmid pTiC58Δ*accR* conferred a hyperconjugal phenotype and very high levels of AAI production. Expression of *traM* required TraR, indicating that transcription of the gene is regulated by the autoinduction system. TraM had no effect on the expression of *traR*, demonstrating that the suppressive effect is not due to repression of the gene encoding the activator. These results suggest that TraM is not a direct transcriptional regulator. Since the suppressive effect is demonstrable only when *traM* is overexpressed with respect to *traR*, we suggest that TraM functions to sequester TraR from the very small amounts of AAI produced under conditions when the agrocinopines are not present.

The regulation of the *lux* operon of *Vibrio fischeri* by autoinduction has established the paradigm for density-dependent control of gene expression (23, 33, 34). In this system, expression of the *lux* genes requires the transcriptional activator LuxR (17). LuxR, in turn, requires as a coinducer a diffusible signal molecule, *N*-(β-ketohexanoyl)-L-homoserine lactone (HSL) (18), also called *Vibrio* autoinducer, VAI (23). The gene, *luxI*, located at the 5′ end of the *lux* operon, is responsible for the production of VAI (20). During growth, *V. fischeri* produces small amounts of VAI which diffuses out of the cells into the culture supernatant (27). When total VAI reaches a certain overall concentration as a function of cellular growth, the autoinducer is believed to interact with LuxR (1), converting it to a functional activator (12). Thus, expression of *lux* genes is dependent upon the cells reaching a critical population density (33).

Recently other biological phenomena, including pathogenicity (26, 36, 40), extracellular enzyme biosynthesis (26, 40), and antibiotic biosynthesis (3, 37), were found to be regulated by autoinduction. In *Agrobacterium tumefaciens*, autoinduction regulates expression of genes required for the conjugal transfer of Ti plasmids (22, 38). Three regions of the nopaline-type Ti plasmid pTiC58 are essential for conjugal transfer (6); expression of genes in the *tra* and *trb* regions requires the transcriptional activator, TraR, encoded in the third (38). TraR is a LuxR homolog and, like the *V. fischeri* activator, requires a diffusible signal molecule, produced by the donor cells, as a coinducer (38). This molecule, now called *Agrobacterium* autoinducer (AAI) (23), is *N*-(β-ketooctanoyl)-L-HSL, a structural analog of VAI (56). Substituted HSLs, collectively called autoinducers (AI), also are produced by other gram-negative bacteria, including *V. harveyi* (10), *Pseudomonas aeruginosa* (36a), *Erwinia carotovora* (40, 49), *Enterobacter agglomerans* (49), *Yersinia enterocolitica* (51), and *Chromobacterium violaceum* (54). HSL autoinducers can differ in the length of and in the nature of the substitution at the β carbon of the acyl side chain (23). The gene, *traI*, is responsible for production of AAI by *A. tumefaciens*, and TraI is a homolog of LuxI (22, 25). Other LuxI homologs responsible for autoinducer synthesis by *P. aeruginosa*, *Erwinia carotovora*, and *Enterobacter agglomerans* have been identified and show significant identities to LuxI and TraI at the amino acid sequence level (23, 25).

The current model for primary regulation of the *V. fischeri* Lux system by autoinduction requires only two components, the transcriptional activator LuxR and the diffusible coinducer VAI (33). We describe here a third regulatory element involved in autoinduction of the Ti plasmid *tra* genes. This element appears to prevent the basal level of TraR present in uninduced cells from activating transcription of the *tra* regulon.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and reagents. The strains of *A. tumefaciens* and *Escherichia coli* and the plasmids used are listed in Table 1. *A. tumefaciens* strains were grown in L broth (LB) (42) or on nutrient agar plates (Difco Laboratories, Detroit, Mich.) at 28°C. *E. coli* cells were grown in LB or on L agar plates at 37°C. AB medium (11) supplemented with 0.2% mannitol or with 1 mM nopaline and 9 mM arginine (6) as the sole carbon source was used as the defined minimal medium for culturing *A. tumefaciens*. When required, antibiotics were added at the following concentrations: for *A. tumefaciens*, tetracycline, 2 μg/ml; carbenicillin, 100 μg/ml; kanamycin, 50 or 100 μg/ml; rifampin, 100 μg/ml; and streptomycin, 200 μg/ml; for *E. coli*, tetracycline 10 μg/ml; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 30 μg/ml. Nopaline and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) were purchased from Sigma Chemical Co. (St. Louis, Mo.), and synthetic *N*-(β-ketooctanoyl)-L-HSL was a

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TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
A. tumefaciens		
NT1	pTiC58-cured strain	53
C58	Wild type, Tra^i	57
C58C1RS	pTiC58-cured strain, Rif^r Str^r	52
NT1(pTiC58Δ*accR*)	*accR* deletion in pTiC58, Tra^c	5
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*)U169	42
S17-1	Pro^- Res^- Mod^+ *recA*; integrated RP4-Tet::Mu-Kan::Tn*7*, Mob^+	45
S17-1(pHoHo1, pSShe)	Amp^r Cm^r, source of Tn*3*HoHo1	46
BL21(DE3)(pLysS)	B strain; F^- *ompT* r_B^- m_B^- *hsdS gal*(λDE3 c*Its*857 *int-1 Sam7 nin-5 lacUV5*-T7 gene 1) Cm^r	48
C2110	Nal^r *polA*	46
2174(pPH1JI)	*met pro* Gm^r Sp^r	7
Plasmids		
pTZ18	Amp^r	32
pET3d	Amp^r, protein expression vector	48
pRK415K	Tet^r Kan^r IncP1α	14
pDSK519	Kan^r IncQ	28
pCP13/B	Tet^r IncP1α	16
pLAFR3	Tet^r IncP1α	47
pSa152	Kan^r Cm^r Str^r IncW	50
pGS9	Kan^r Cm^r	44
pTHB58	Tet^r, *Bam*HI partial cosmid clone of pTiC58 carrying the *tra*/*oriT* region and *traM*	24
pOW1	2.4-kb *Eco*RI fragment 26 encoding *accR* from pTiC58 cloned into pRK415K	5
pOC1	2.4-kb *Eco*RI fragment 26 encoding *accR* deletion allele from pTiC58Δ*accR* cloned into pRK415K	5
pSVB33	1.8-kb *Eco*RI fragment encoding *traR* from pTiC58 cloned into pSa152	38
pHS3	1.6-kb *Bam*HI-*Sal*I fragment cloned into pTZ18	This study
pMA1	412-bp *Nco*I-*Bam*HI fragment amplified from pHS3 cloned into pET3d	This study
pMP1	10-kb *Hin*dIII fragment 3 cloned into pCP13/B	This study
pCHT10	pTiC58*traM*::*lacZ10*	This study
pKHT10	pTiC58Δ*accRtraM*::*lacZ10*	This study
pHS10	10-kb *Hin*dIII fragment 3 cloned into pLAFR3	This study
pHS11	*Sst*I deletion derivative of pHS10	This study
pHS14	*traM*::*nptII* from pHS11	This study
pMB1	*traM*::*bla* from pHS11	This study
pPLE33	1.8-kb *Eco*RI fragment encoding *traR* from pTiC58 cloned into pDSK519	This study
pDCB24	1.9-kb *Bam*HI fragment 24 cloned into pPLE33	This study
pYZ1	1.9-kb *Bam*HI fragment 24 cloned into pRK415K	This study
pDCBP	1.1-kb *Bam*HI-*Pst*I fragment from pHS3 cloned into pPLE33	This study
pDCΔB	1.1-kb *Bam*HI fragment from pHS11 cloned into pPLE33	This study
pDCI41	pTHB58::Tn*3*HoHo1-I41, *tra*::*lacZI41*	This study
pDCII24	pTHB58::Tn*3*HoHo1-II24, *tra*::*lacZII24*	This study
pH4II24	*Hin*dIII fragment 4 containing Tn*3*HoHo1 from pDCI41 cloned into pCP13/B	This study
pH4I41	*Hin*dIII fragment 4 containing Tn*3*HoHo1 from pDCII24 cloned into pCP13/B	This study
pDH4I41	*Hin*dIII fragment 4 containing Tn*3*HoHo1 from pDCI41 cloned into pDSK519	This study
pDCCI41	pTiC58*tra*::*lacZI41*	This study
pDCCII24	pTiC58*tra*::*lacZII24*	This study
pDCKI41	pTiC58Δ*accR*, *tra*::*lacZI41*	This study
pDCKII24	pTiC58Δ*accR*, *tra*::*lacZII24*	This study
pCMI41	pTiC58*tra*::*lacZI41*, *traM*::*nptII*	This study
pCMII24	pTiC58*tra*::*lacZII24*, *traM*::*nptII*	This study
pKMI41	pTiC58Δ*accR*, *tra*::*lacZI41 traM*::*nptII*	This study
pKMII24	pTiC58Δ*accR*, *tra*::*lacZII24 traM*::*nptII*	This study
pCMA1	pTiC58*traM*::*nptII*	This study
pKMA1	pTiC58Δ*accR*, *traM*::*nptII*	This study
pTiC12	pTiC58*traR*::*lacZ12*	39
pTiK12	pTiC58Δ*accR*, *traR*::*lacZ12*	39
pTiC12M	pTiC58*traR*::*lacZ12*, *traM*::*bla*	This study
pTiK12M	pTiC58Δ*accR*, *traR*::*lacZ12 traM*::*bla*	This study

[a] Tra^i, conjugal transfer inducible; Tra^c, conjugal transfer constitutive; Nal^r, nalidixic acid resistant, Amp^r, ampicillin resistant; Gm^r, gentamicin resistant; Sp^r, spectinomycin resistant; Tet^r, tetracycline resistant; Kan^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Str^r, streptomycin resistant.

generous gift from David Lynn (University of Chicago). Crude AAI was prepared from culture supernatants of *A. tumefaciens* NT1(pTiC58Δ*accR*) grown overnight in AB-mannitol medium as described previously (38).

DNA manipulations. Plasmid DNA was isolated by the alkaline lysis method (42). Standard recombinant DNA techniques were used as described by Sambrook et al. (42). Restriction digestions were done as described by the manufacturer (GIBCO BRL, Gaithersburg, Md.), and electrophoresis in agarose gels was performed in Tris-borate-EDTA buffer (42).

Tn*3*HoHo1 mutagenesis. The cosmid clone pTHB58 and the subclone pMP1, which contains *Hin*dIII fragment 3 in pCP13/B (Table 1), were mutagenized with Tn*3*HoHo1 as described previously (46). Mutations were homogenotized into pTiC58 (wild type, transfer inducible) and into pTiC58Δ*accR* (transfer constitu-

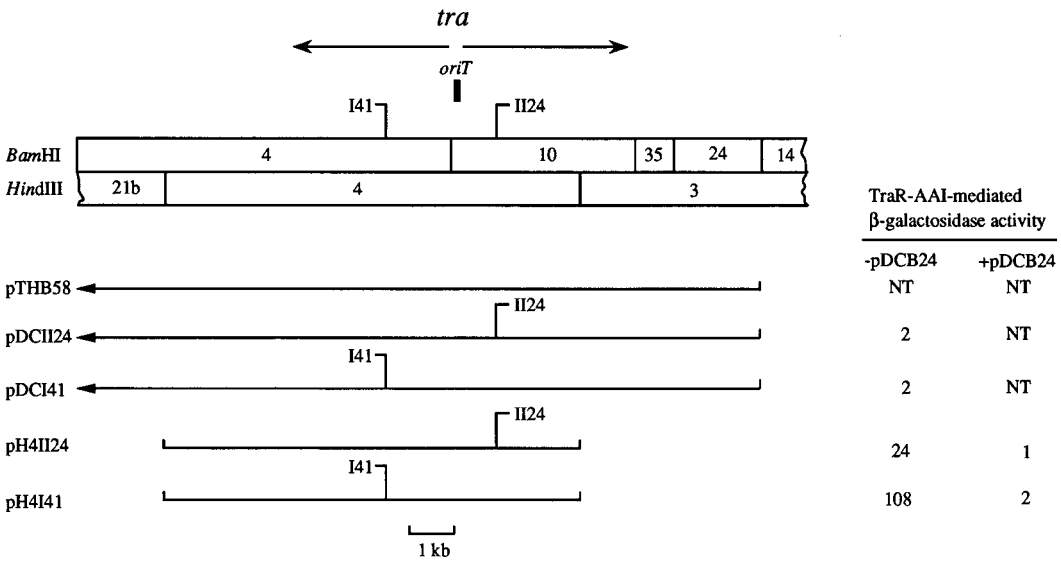

FIG. 1. Physical map of the *tra* region of pTiC58. The positions of the two Tn*3*HoHo1-generated *tra*::*lacZ* fusions, I41 and II24, are indicated by the vertical lines, with the crossbars showing the direction of transcription reported by each fusion. TraR-AAI-mediated expression from the reporter fusions on the different clones and subclones is tabulated at the right. pDCB24 is pPLE33 containing *Bam*HI fragment 24, which encodes *traM*. pDCII24 and pDCI41 each contain the region represented by *Bam*HI fragment 24 and therefore themselves encode *traM*. Synthetic AAI was added to cultures to a final concentration of 25 nM. β-Galactosidase activity, expressed as units per 10^9 CFU, was measured as described in Materials and Methods. NT, not tested.

tive [Tra^c]) as described by Ruvkun and Ausubel (41). Homogenotizations were confirmed by restriction enzyme analysis of isolated Ti plasmid DNA.

β-Galactosidase assay. β-Galactosidase activity was determined qualitatively on AB-mannitol agar medium containing 40 μg of X-Gal per ml. For quantitative assays, strains were grown in AB-mannitol medium overnight at 28°C. Cells were washed and diluted 10- to 20-fold in the same medium, and the incubation continued until the optical density at 600 nm reached 0.6. When required, the cultures were divided and samples were induced with AAI. β-Galactosidase activity was measured and expressed as units per 10^9 CFU as described previously (46).

AAI and conjugal transfer assays. Production of AAI by *Agrobacterium* strains was determined qualitatively on AB-mannitol agar plates containing X-Gal, using the reporter strain NT1(pJM749, pSVB33) as described previously (38). Frequencies of conjugal transfer for various Ti plasmids were measured by the spot mating method and expressed as transconjugants per input donor as described previously (6).

PCR and sequencing. The *nptII* (neomycin phosphotransferase II) and *bla* (β-lactamase) genes were amplified, using pGS9 (44) and pTZ18 (32) DNA, respectively, as templates, by PCR to generate *nptII* and *bla* cassettes flanked by *Sst*I sites. PCR primers for the *nptII* gene amplification were Km3 (5′-CCGAGCTCCGAACCCCAGAGTCCCGCTC-3′) and Km4 (5′-CGGAGCTCGCAAAGAGAAAGCAGGTAGCTTGCAGT-3′) (*Sst*I sites are underlined). PCR primers for the *bla* gene amplification were Bla1(5′-CCGAGCTCAAAGGATCTTCACCTAGATC-3′) and Bla2 (5′-GCGAGCTCGGAAATGTGCGCGGAACCCC-3′) (*Sst*I sites are underlined). Primer oligonucleotides were obtained from the Genetic Engineering Facility at the University of Illinois. Target DNA was amplified by using a programmable thermal controller (MJ Research, Inc., Watertown, Mass.) and the GeneAmp kit with UlTma DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). Reaction conditions were as follows: melting temperature, 92°C; annealing temperature, 50°C; polymerization temperature, 72°C; and 25 cycles.

DNA fragments were sequenced by the dideoxy method (43), using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Co., Cleveland, Ohio). DNA sequences were analyzed by using the DNA Strider program (30), MacPROT program (31), and BLAST protocols (2) to search databases. The sites of selected Tn*3*HoHo1 insertions were determined by using the primer Tn3LEAD (5′-GTCAGAGGCAGAAAAC-3′), homologous to the 5′ end of the *tufB* gene sequence of Tn*3*HoHo1 (46).

Protein expression. A 412-bp *Nco*I-*Bam*HI fragment containing the *traM* open reading frame (ORF) was amplified by using primers TraM1 (GCCCATGGAATCGGAAGATGCAACATTGACG) and TraM2 (CGGGATCCTCCGATGAAGATAGCGGGTCTGAG) by PCR as described above and cloned into pET3d (48). The cloned fragment was sequenced as described above to confirm the absence of any PCR-induced amino acid sequence alterations. The recombinant plasmid, pMA1, was transformed into *E. coli* BL21(DE3)(pLysS) (48). Cells were inoculated into 2 ml of LB containing chloramphenicol and ampicillin and incubated for 8 h at 37°C. Cells were stored overnight at 4°C, diluted 50-fold with LB, and incubated for 2 h at 37°C. For induction, isopropyl-β-D-thiogalactoside (IPTG; GIBCO BRL) was added at a final concentration of 1 mM, and incubation continued for an additional 4 h. Cells were collected and stored at −70°C. Total protein was prepared as described by the manufacturer (Novagen, Inc., Madison, Wis.) and subjected to electrophoresis in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Gels were stained with Coomassie brilliant blue R250 (Bio-Rad).

Nucleotide sequence accession number. DNA sequences have been deposited in the GenBank database under accession number L34744.

RESULTS

Response of *tra*::*lacZI41* and *tra*::*lacZII24* to TraR and exogenous AAI. In a study to determine how TraR and AAI influence expression of *tra* genes, we examined two *lacZ* fusions, I41 and II24, located in the *tra* region of pTiC58 (Fig. 1). These fusions report expression of two *tra* operons transcribed divergently from the *oriT*/*nic* region (13). Strains NT1(pDCI41) and NT1(pDCII24) also carrying *traR* cloned in the IncW vector pSa152 (pSVB33) were seeded in 0.7% AB-mannitol soft agar containing X-Gal. When crude AAI was spotted onto these plates, no significant β-galactosidase activity was detected (data not shown). This result, which is contrary to results observed for another *tra*::*lacZ* fusion, pJM749, carried on the same cosmid clone (38), was confirmed by quantitative assays for β-galactosidase activity (Table 2). When strains of NT1 carrying other independent *tra*::*lacZ* fusions and pSVB33 were tested as described above, *tra* expression was similarly uninducible by exogenous AAI (data not shown). However, inhibition was not observed in strains NT1(pDCI41) and NT1 (pDCII24) carrying pPLE33 in which *traR* is cloned in the high-copy-number IncQ vector pDSK519 (Table 2). The suppressive effect could not be overcome by adding additional exogenous AAI. AAI induced half-maximal reporter gene activity when added at a concentration of about 3 nM to a strain expressing *traR* from pPLE33 (Fig. 2A). However, addition of AAI to concentrations up to 100 μM failed to induce the *tra*::*lacZ* fusion in the strain expressing *traR* from pSVB33.

We reasoned that the inhibitory function probably was

TABLE 2. Responses of *tra* gene expression to TraR and exogenous AAI[a]

tra::*lacZ* reporter[b]	β-Galactosidase activity (U/10^9 CFU) in presence of plasmid:		
	None	pSVB33	pPLE33
pDCII24	2	2	17
pDCI41	2	2	48
pH4II24	2	10	NT[c]
pH4I41	2	75	NT

[a] 25 nM AAI was added to each culture.
[b] Each fusion carried on a multicopy plasmid.
[c] NT, not tested.

encoded on one end or the other of the insert in pTHB58. To test this, we constructed recombinant plasmids pH4I41 and pH4II24 by cloning *Hin*dIII fragment 4 from pDCI41 and pDCII24, respectively, into pCP13/B (Table 1; Fig. 1). These two subclones, which in each case contain the entire *lacZ* fusion region from Tn*3*HoHo1, were tested as reporter clones in *trans* to pSVB33 in strain NT1. Each strain produced a substantial amount of β-galactosidase activity following incubation with AAI, indicating that expression of genes in *tra* now responds to the autoinducer (Table 2).

Identification of *traM*. These observations suggested that some function encoded by pTHB58, but lacking in the *Hin*dIII fragment 4 subclones, suppresses *tra* expression mediated by TraR and AAI. To localize this determinant, a series of subclones of pTHB58 were tested for the ability to inhibit expression of the *tra*::*lacZ* fusion in pH4I41 mediated by TraR and exogenous AAI. We used pPLE33 as the vector for these clonings in order to simultaneously provide a source of TraR. One clone, pDCB24, which contains *Bam*HI fragment 24 of pTiC58 conferred a suppressive phenotype. When strains NT1(pH4I41, pDCB24) and NT1(pH4II24, pDCB24) were tested for *tra*::*lacZ* expression in the presence of 25 nM AAI, no significant β-galactosidase activity was detected (Fig. 1). A 1.1-kb *Bam*HI-*Sal*I fragment from *Bam*HI fragment 24 was subcloned into pTZ18 to construct pHS3 (Table 1). pHS3 was digested with *Bam*HI and *Pst*I, the site for which is directly adjacent to the *Sal*I site in the pTZ18 polylinker, to release the 1.1-kb *Bam*HI-*Sal*I fragment. This *Bam*HI-*Pst*I fragment was cloned into pPLE33, resulting in pDCBP (Table 1). This subclone suppressed the TraR-AAI-mediated induction of *tra*::*lacZ* fusions in strains NT1(pH4I41) and NT1(pH4II24) (Table 3). Again, the suppression effect could not be overcome by adding excess AAI (data not shown).

The DNA sequence of the 1.1-kb *Bam*HI-*Sal*I fragment was determined (Fig. 3). This fragment contains a single ORF, which we name *traM*, which could encode a 102-amino-acid protein of 11,219 Da. The direction of transcription is clockwise on pTiC58. The ORF is preceded by a reasonable ribosomal binding site (CCAGGG), a 9-bp perfect direct repeat sequence located 46 bases upstream from the putative start codon, and two sets of sequences showing similarities to canonical −10 and −35 promoter elements (Fig. 3). The hydropathy plot of TraM generated by using the Kyte-Doolittle algorithm (29) indicated that the carboxy terminus of the protein is highly hydrophobic. No significant relatedness was detected between TraM and other protein sequences present in the databases.

We examined the relationship between the suppressive effect and the *traM* ORF by deleting the two *Sst*I fragments from *Hin*dIII fragment 3 of pHS10 (Fig. 4). This produced pHS11,

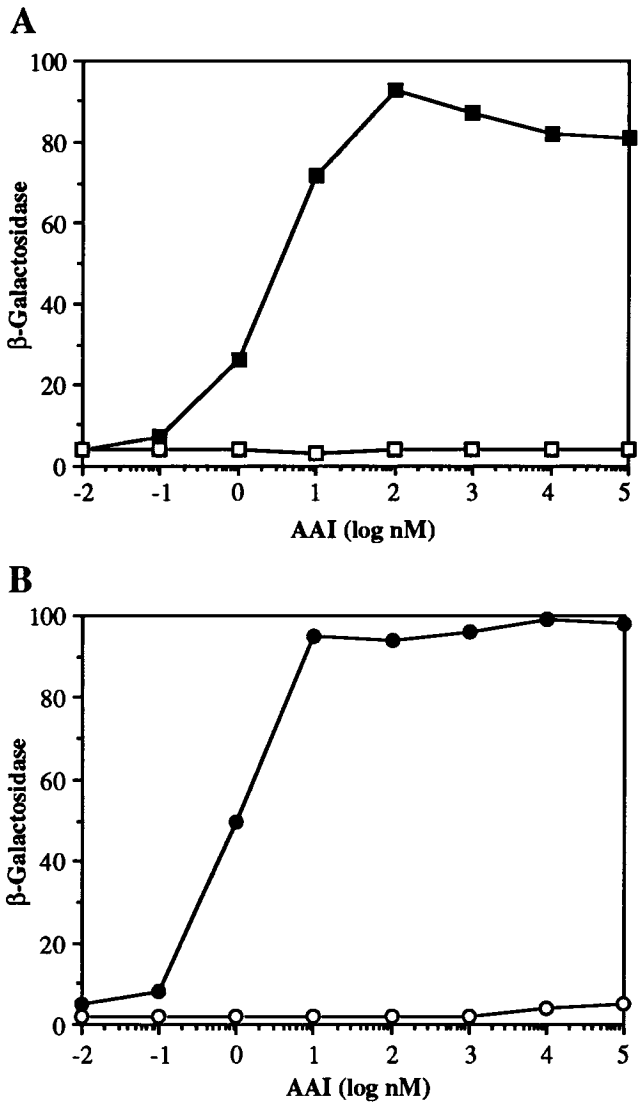

FIG. 2. Influence of TraM and TraR on AAI-mediated inducibility of *tra* genes. Cells were grown in AB-mannitol medium to a density of about 2×10^7 CFU/ml. Synthetic AAI was added at the concentrations shown to samples of these cultures, and incubation was continued for 6 h at 28°C. Cells were harvested, and β-galactosidase levels, expressed as units per 10^9 CFU, were determined as described in Materials and Methods. (A) Influence of *traR* copy number. Indicator cells contained the *traM*$^+$ reporter plasmid pDCI41 and pPLE33, the high-copy-number *traR* clone (■), or pSVB33, the low-copy-number *traR* clone (□). (B) Influence of a *traM* mutation. Indicator cells contained the reporter plasmid pDH4I41, which lacks *traM*, and pHS10 (*traM*$^+$ *traR*$^+$) (○) or pHS11 (*traM traR*$^+$) (●).

which encodes only 12 amino acids of the putative TraM protein (Fig. 3). We also subcloned the modified *Bam*HI fragment 24 from pHS11 into pPLE33 to construct pDCΔB. Both plasmids failed to suppress *tra* expression mediated by TraR and AAI, indicating that the *Sst*I deletion in the ORF abolishes the *traM* phenotype (Fig. 4; Table 3).

***traM* encodes an 11.2-kDa protein.** The *traM* ORF was subcloned into the *E. coli* expression vector pET3d as described in Materials and Methods to produce pMA1. When *E. coli* BL21(DE3)(pLysS, pMA1) was induced with 1 mM IPTG, a novel insert-specific protein of approximately 11 kDa was produced in large amounts (Fig. 5).

A mutation in *traM* derepresses conjugal transfer of wild-

TABLE 3. Mutation in *traM* alleviates suppression of TraR-AAI-mediated expression of *tra* genes

Genotype[a]	β-Galactosidase activity (U/10^9 CFU) from[b]:	
	lacZII24[c]	*lacZI41*[d]
traR$^+$ *traM*$^+$	1	2
traR$^+$ *traM*	19	114

[a] Wild-type allele carried on pDCBP; mutant allele carried on pDCΔB.
[b] AAI was supplied exogenously as described in Materials and Methods.
[c] Carried on pH4II24.
[d] Carried on pH4I41.

type pTiC58. We examined the effects of disruption in the *traM* region of pTiC58 by transposon mutagenesis. Eleven independent Tn*3*HoHo1 insertions mapping to the *Hin*dIII fragment 3 insert in pMP1 (Fig. 4) were isolated, and each insertion was marker exchanged into pTiC58 and pTiC58Δ*accR*. Strain NT1(pTiC58Δ*accR*) carrying Tn*3*HoHo1 insertion 44 located in *traR* is transfer deficient (Tra$^-$) as expected. Strains of NT1 carrying pTiC58Δ*accR* derivatives with other Tn*3*HoHo1 insertions mapping within this region all are Tra$^+$ (Fig. 4). Strains carrying ten of the independent Tn*3*HoHo1 insertions marker exchanged into wild-type pTiC58 are phenotypically Tra$^-$ when mated without prior induction with agrocinopines (Fig. 4). However, strain NT1(pCHT10), which carries Tn*3*Ho Ho1 insertion 10 in pTiC58, is Tra^c (Fig. 4). Also, unlike its wild-type parent, strain NT1(pCHT10) produces AAI without need for induction with agrocinopines (Table 4). The same insertion in pTiC58Δ*accR*, pKHT10, resulted in a strain that is hyperconjugal and produces detectably more AAI than NT1(pTiC58Δ*accR*) (Table 4). Analysis of β-galactosidase activities expressed from the 11 independent insertions indicated that there are at least two transcriptional units in the region (Fig. 4). Strains of NT1 harboring five insertions, 5, 17, 6, 14, and 10, oriented clockwise, and two insertions, 3 and 36, oriented anticlockwise on pTiC58Δ*accR*, produced β-galactosidase activity as monitored on AB-mannitol medium containing X-Gal (Fig. 4). None of the insertions marker exchanged into otherwise wild-type pTiC58 produced β-galactosidase activity under these same conditions (Fig. 4).

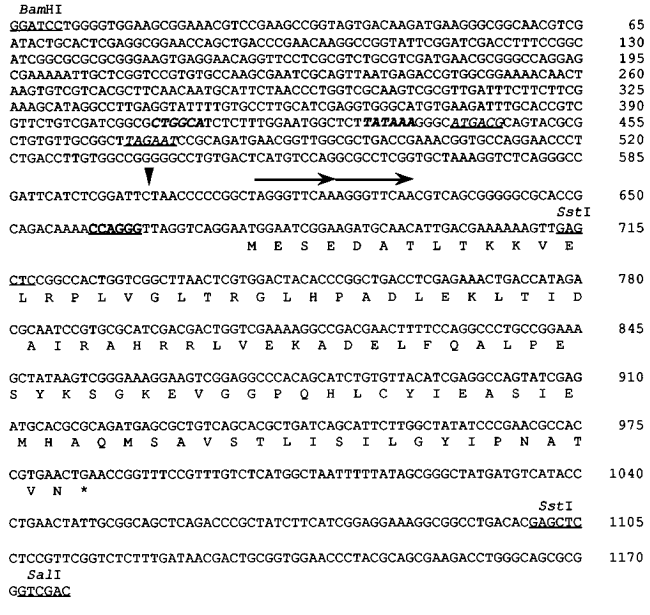

FIG. 3. DNA and predicted amino acid sequences of *traM* contained in the 1.1-kb *Bam*HI-*Sal*I fragment. A putative ribosome binding site is in boldface and underlined. The vertical arrow indicates the location of Tn*3*HoHo1 insertion 10. The two horizontal arrows mark the 9-bp direct repeat sequence. One set of potential promoter sequences is shown in boldface italics, while the other is shown in underlined italics.

Sequence analysis located Tn*3*HoHo1 insertion 10 to a site 75 bases upstream from the putative start codon of *traM* (Fig. 3). Since this insertion is located in the presumed promoter region of *traM*, we constructed a *traM* ORF disruption mutant by inserting an *nptII* gene cassette into the single *Sst*I site of pHS11, producing pHS14 (Fig. 4). This construct was marker exchanged into pTiC58 and pTiC58Δ*accR*, producing pCMA1 and pKMA1, respectively (Table 1). Strain NT1(pCMA1) produces AAI and is Tra^c, giving a conjugal transfer frequency similar to that of pTiC58Δ*accR* (Table 4). Strain NT1(pKMA1) produces more AAI than does strain NT1(pTiC58Δ*accR*), and pKMA1 transfers at a frequency 10-fold higher than that of pTiC58Δ*accR* (Table 4). These phenotypes are indistinguishable from those exhibited by the same Ti plasmids harboring Tn*3*HoHo1 insertion 10 located in the upstream untranslated region of *traM.*

Effect of a *traM* null mutation on *tra* expression in pTiC58. The *tra*::*lacZI41* and *tra*::*lacZII24* fusions were marker exchanged into pTiC58 and pTiC58Δ*accR*, producing pDCCI41, pDCCII24, pDCKI41, and pDCKII24, respectively (Table 1). Derivatives of each of these *tra* reporter Ti plasmids containing the *traM* mutation then were constructed by marker exchanges using pHS14 (*traM*::*nptII*; Table 1). This produced pCMI41 (*accR*$^+$ *tra*::*lacZI41 traM*::*nptII*), pCMII24 (*accR*$^+$ *tra*::*lacZII24 traM*::*nptII*), pKMI41 (*accR tra*::*lacZII24 traM*::*nptII*), and pKMII24 (*accR tra*::*lacZII24 traM*::*nptII*) (Table 1).

In the *traM*$^+$ backgrounds, expression of the *tra*::*lacZ* reporters is controlled by AccR, being substantially higher in strains mutant at *accR* (Table 5 and reference 5). β-Galactosidase levels in the *traM accR*$^+$ strains NT1(pCMI41) and NT1(pCMII24) were 3- to 10-fold higher than in their *traM*$^+$ *accR*$^+$ parents (Table 5). This result is consistent with the observation that a *traM accR*$^+$ Ti plasmid is Tra^c (Table 4). Strains harboring *traM accR* Ti plasmids showed levels of β-galactosidase activities similar to those of the *traM*$^+$ *accR* strains NT1(pDCKI41) and NT1(pDCKII24) (Table 5).

TraM prevents AAI-mediated autoinduction in cells expressing basal levels of TraR. Suppression by TraM can be overcome by increasing the levels of TraR, a condition that results when cells encounter the conjugal opines (Fig. 2A). This finding suggests that under normal conditions, TraM inhibits *tra* gene activation by the basal levels of TraR present in cells that have not been induced by the opine signals. However, these experiments were performed with strains in which *traR* and *traM* are expressed from separate replicons that differ in copy number. To approach this question, we constructed two plasmids that contain the entire *traR-traM* region of pTiC58 in its native configuration. As such, the two genes each contain their putative upstream promoter regions. One plasmid, pHS10, contains wild-type *traM*, while the second, pHS11, contains the *Sst*I deletion derivative of *traM* (Fig. 4). TraR-mediated activation of expression of the *tra*::*lacZ* reporter was not detected in cells harboring the *traM*$^+$ derivative, even when incubated with as much as 100 μM AAI (Fig. 2B). However, the reporter fusion in the *traM* mutant responded to the addition of the AAI. Moreover, the concentration of AAI required for half-maximal induction was similar to that required for induction in the strain expressing *traR* from the high-copy-number clone pPLE33 (Fig. 2A). These results suggests that *traR* is expressed in the absence of the conjugal opines but that

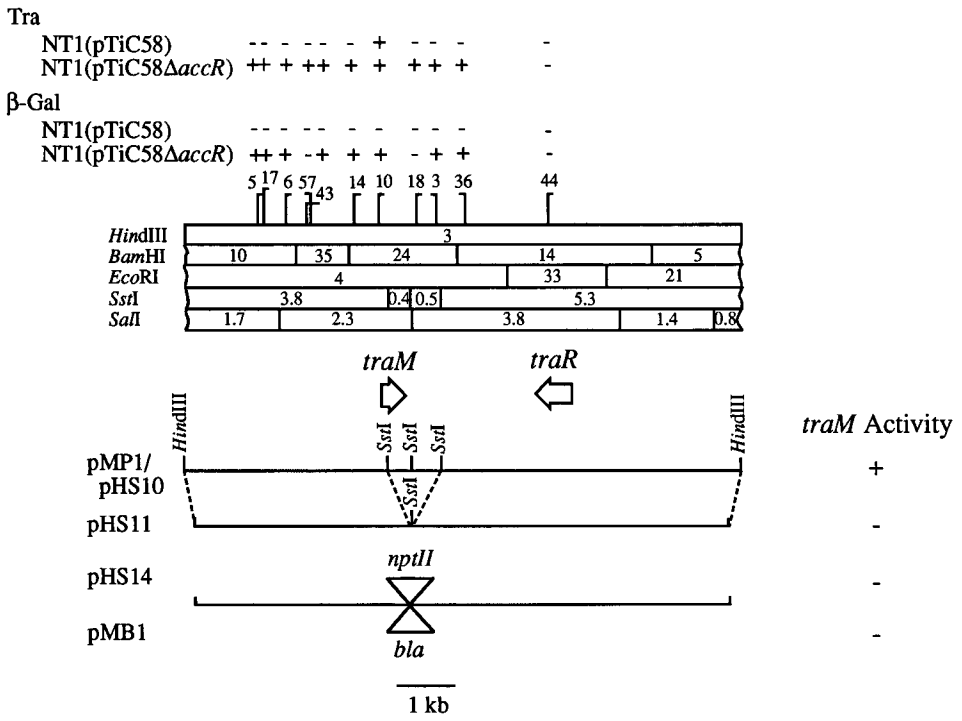

FIG. 4. Insertion and deletion analysis of the *traM-traR* region of pTiC58. The maps show positions of Tn*3*HoHo1 insertions (vertical lines) and deletion and insertion alterations in the *traM* region of *Hin*dIII fragment 3. Crossbars on each insertion indicate the transcriptional direction reported by the Tn*3*HoHo1 *lacZ* gene. Each Tn*3*HoHo1 insertion was marker exchanged into pTiC58 and pTiC58Δ*accR*. β-Galactosidase activity, as determined on X-Gal plates, is indicated (+ or −) for each of the Tn*3*HoHo1 insertions. Conjugal transfer ability of each marker-exchanged mutant was assayed as described in Materials and Methods. +, constitutive conjugal transfer; −, conjugation frequency of $<10^{-9}$ under noninducing conditions. The two open arrows indicate the locations and transcriptional orientations of *traR* and *traM*. *traM* activity was determined as described in Materials and Methods, using NT1(pH4I41) as the indicator strain. +, suppresses *tra* gene expression mediated by TraR and exogenous AAI; −, suppression of *tra* gene expression was not observed. *Sst*I and *Sal*I fragments are identified by their sizes (in kilobases) as determined by electrophoretic mobilities in agarose gels.

the levels of the activator produced under these conditions are insufficient to overcome the suppressive effect of TraM.

Expression of *traM* is regulated by AccR through TraR. Analysis of expression of the *traM*::*lacZ10* reporter fusion indicates that *traM* is expressed in strain NT1(pTiC58Δ*accR*) but not in strain NT1(pTiC58) (Fig. 4; Table 6), which suggests that expression of *traM* is regulated by AccR. To test this, pOW1 and pOC1, containing wild-type and mutant alleles of *accR*, respectively (5), were introduced into strain NT1 (pKHT10). Expression of *traM*, as monitored by β-galactosidase activity, was repressed in strain NT1(pKHT10, pOW1) but not in strain NT1(pKHT10, pOC1) (Table 6). Expression of *traM* in the *accR*$^+$ strain NT1(pCHT10) was derepressed by incubating cells with agrocinopines (Table 6). These findings indicate that *traM* expression is controlled by AccR.

Expression of *traR*, the gene that encodes the direct activator of *tra* genes, is itself regulated by AccR (39). Thus, it is possible that AccR regulates *traM* expression indirectly

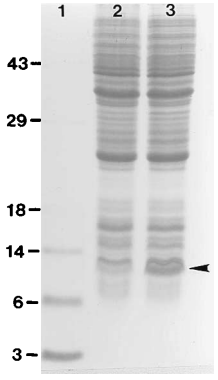

FIG. 5. Expression of TraM in *E. coli*. Total proteins were prepared from *E. coli* BL21(DE3)(pLysS, pMA1) as described in Materials and Methods and separated in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The 11.2-kDa TraM protein was expressed when cells were induced with 1 mM IPTG. Lane 1, low-molecular-weight size standard (Bio-Rad); lane 2, total protein profiles of uninduced cells; lane 3, total protein profiles of induced cells. The arrow points to the 11.2-kDa TraM protein. Sizes are indicated in kilodaltons.

TABLE 4. Effects of a *traM* mutation on conjugal transfer and production of AAI

Strain	Ti plasmid genotype	AAI production[a]	Conjugation frequency[b]
NT1(pTiC58)	Wild type	−	$<1.2 \times 10^{-9}$
NT1(pTiC58Δ*accR*)	*accR traM*$^+$	++	10^{-4}
NT1(pCHT10)	*accR*$^+$ *traM*::*lacZ10*	+	10^{-6}
NT1(pKHT10)	*accR traM*::*lacZ10*	+++	10^{-3}
NT1(pCMA1)	*accR*$^+$ *traM*::*nptII*	++	10^{-4}
NT1(pKMA1)	*accR traM*::*nptII*	+++	10^{-3}

[a] AAI production was evaluated on AB minimal medium containing indicator cells and X-Gal. −, no diffuse blue zones; ++, diffuse blue zone equivalent to that produced by NT1(pTiC58Δ*accR*); +, diffuse blue zone evident but less than that produced by NT1(pTiC58Δ*accR*); +++, diffuse blue zone larger than that produced by NT1(pTiC58Δ*accR*).

[b] Expressed as number of transconjugants per input donor.

TABLE 5. Effects of *traM* and *accR* mutations on *tra* gene expression

Reporter fusion[a]	*accR* genotype	β-Galactosidase activity (U/10^9 CFU)		Fold difference
		traM$^+$	*traM*	
tra::*lacZII24*	*accR*$^+$	3	10	3.3
	accR	40	41	
tra::*lacZI41*	*accR*$^+$	3	39	13.0
	accR	83	106	1.3

[a] Each reporter fusion contained in pTiC58 (*accR*$^+$) and pTiC58Δ*accR* (*accR*).

TABLE 7. TraR activates *traM* expression in the absence of TraM

traR or *traM*	β-Galactosidase activity (U/10^9 CFU) from *traM*::*lacZ10*	
	accR$^+$[a]	*accR*[b]
traR single copy	3	30
traR multicopy[c]	22	25
traM multicopy[d]	5	9

[a] pCHT10, *traM*::*lacZ10 accR*$^+$.
[b] pKHT10, *traM*::*lacZ10* Δ*accR*.
[c] Made multicopy for *traR* by introducing pPLE33 into the tester strain.
[d] Made multicopy for *traM* by introducing pYZ1 into the tester strain.

through TraR. To test this, we introduced pPLE33 into strain NT1(pCHT10). This plasmid expresses *traR*, resulting in the production of activator in an *accR*$^+$ strain (15, 38). β-Galactosidase activity from the *traM*::*lacZ* fusion in strain NT1 (pCHT10, pPLE33) was sevenfold higher than that in strain NT1(pCHT10) (Table 7). This result indicates that *traM* expression is activated by TraR and that repression of *traM* expression by AccR is indirect.

Expression of *traM* is repressed in pTiC58Δ*accR* when TraM is supplied in multicopy. When plasmid pYZ1 carrying *Bam*HI fragment 24 in pRK415K was introduced into strain NT1(pKHT10), expression of *traM*, as monitored by β-galactosidase activity from the *traM*::*lacZ* fusion, was repressed (Table 7). Clone pYZ1 had no effect on the repressed levels of *traM* expression in strain NT1(pCHT10) (Table 7).

TraM does not affect expression of *traR*. We considered the possibility that TraM exerts its modulating activity by regulating the expression of *traR*. To test this, a *traM*::*bla* mutation was marker exchanged into strains NT1(pTiC12) and NT1 (pTiK12), which carry a *traR*::*lacZ* fusion in pTiC58 and pTiC 58Δ*accR*, respectively (39), producing strains NT1(pTiC12M) and NT1(pTiK12M). β-Galactosidase levels in these two *traM* mutants were similar to levels observed in their *traM*$^+$ parents (data not shown), indicating that TraM does not influence *traR* expression.

DISCUSSION

Conjugal transfer of Ti plasmids is regulated by autoinduction mediated by the transcriptional activator, TraR, and the substituted HSL coinducer, AAI (22, 25, 38). In turn, this system is itself regulated; activation of *tra* gene expression by TraR and AAI occurs only when *Agrobacterium* cells encounter specific signals, the conjugal opines, produced by crown gall tumors (19). In pTiC58, this opine-responsive control is mediated by the transcriptional repressor, AccR (5).

We report here the identification of a new gene, *traM*, that negatively influences the TraR-AAI-mediated expression of genes in the *tra* region of pTiC58. This regulatory gene, which is located between the *traAF* operon and *traR* on the Ti plasmid, could encode an 11.2-kDa protein, TraM. This is consistent with the size of a novel protein produced by an *E. coli* strain harboring a clone in which the *traM* ORF is expressed from the T7 promoter (Fig. 5).

Expression of *traM* requires TraR (Table 7). The gene is expressed at relatively high levels from pTiC58Δ*accR*, which constitutively produces TraR (38, 39), and also from wild-type pTiC58 (*accR*$^+$) when a functional *traR* clone is expressed in *trans* (Tables 6 and 7). Since expression of *traR* is itself regulated by AccR (38), *traM* is under the control of the opine regulon. This conclusion is consistent with two observations. First, expression of *traM* from pTiC58Δ*accR* is repressed by supplying a copy of wild-type *accR* in *trans* (Table 6). Second, the *traM*::*lacZ* fusion carried on wild-type pTiC58 is induced when a strain harboring this plasmid is incubated with agrocinopines A and B (Table 6).

The promoter regions of the TraR-AAI-dependent *tra* and *trb* operons of pTiC58 each contain an 18-bp inverted repeat called the Tra box (25). This repeat structure forms a conserved family with similar inverted repeats located in the promoter regions of the *lux* operon of *V. fischeri* and the *lasB* gene of *P. aeruginosa* (8, 23, 25). In the *lux* and *tra* systems, these repeats are required for induction of expression of the downstream genes by their cognate activator-HSL-AI pairs (17, 23). However, there is no recognizable Tra box in the region directly upstream of *traM* (Fig. 2). This finding suggests that TraR can activate transcription from promoters that do not contain a canonical Tra box. Alternatively, it is possible that the inducible *traM* expression results from TraR-mediated activation of transcription from the Tra box-containing promoter located in *tra*. The gene is transcribed in the same direction as the rightward reading *traAF* operon (reference 13 and Fig. 1). However, except for insertion 10, none of Tn*3*HoHo1 insertions mapping between *traAF* and *traM* have any effect on conjugal transfer of pTiC58. If *traM* is expressed as part of the *traAF* operon, we would expect at least some of these insertions to confer a Tra^c phenotype due to polarity on this gene. The region upstream of *traM* contains two potential −10 and −35 elements as well as a Shine-Dalgarno-like sequence upstream of the putative ATG initiation codon. In addition, there is a 9-bp perfect direct repeat sequence located 46 bp upstream of the gene. The significance of this repeat sequence, if any, is not known.

Mutations in *traM*, either in the upstream untranslated region or in the structural gene, result in Ti plasmids that are hyperconjugal. In wild-type pTiC58, *traM* mutations confer a Tra^c phenotype, and strains harboring this mutant Ti plasmid produce easily detectable amounts of AAI even in the absence

TABLE 6. Expression of *traM* is controlled by AccR and agrocinopines

Strain	Genotype	β-Galactosidase activity (U/10^9 CFU)	
		−Agrocinopines	+Agrocinopines
NT1(pCHT10)	*traM*::*lacZ10 accR*$^+$	3	18
NT1(pKHT10)	*traM*::*lacZ10 accR*	30	NT[a]
NT1(pKHT10, pOW1)	*traM*::*lacZ10 accR*/*accR*$^+$	7	NT
NT1(pKHT10, pOC1)	*traM*::*lacZ10 accR*/*accR*	27	NT

[a] NT, not tested.

of induction with the conjugal opine. With respect to conjugal transfer functions, this phenotype is similar to that exhibited by the spontaneous Tra^c Ti plasmid mutant, pTiC58Δ*accR* (5). However, the *traM* mutant of pTiC58 still is regulated for expression of *acc*, indicating that the mutation affects the regulatory circuit downstream of AccR (results not shown). In the Tra^c Ti plasmid pTiC58Δ*accR*, *traM* mutations result in constitutive transfer frequencies 10-fold higher than those seen in strains harboring the parent plasmid (Table 4). Moreover, these strains produce even more AAI than do those containing pTiC58Δ*accR*. These phenotypes are consistent with our analysis of the effect of *traM* on expression of the two divergently transcribed *tra* operons (Fig. 1). In pTiC58, which is wild type for both *accR* and *traR*, mutations in *traM* result in a 3- to 13-fold increase in the transcription of the two *tra* reporters over basal levels (Table 5). However, elevated levels of *tra* expression are three to four times lower in *accR*$^+$ *traM* strains compared with the same fusions in the *traM*$^+$ Tra^c Ti plasmid, pTiC58Δ*accR* (Table 5). These results are consistent with the levels of conjugal transfer observed for the two strains and indicate that mutations in *traM* cannot fully overcome the need for induced expression of *traR* (Table 4).

These results suggest that TraM serves to modulate the TraR-AAI-mediated autoinduction system. We first identified *traM* by virtue of its suppressive effect on the expression of *tra* genes carried on recombinant clones (Fig. 1; Table 2). It is possible that TraM is itself a transcriptional repressor, acting to inhibit expression of *traR* or of the *tra* or *trb* operons. This seems to us to be unlikely. The TraM protein does not contain any sequence motifs known to interact with DNA. Furthermore, mutations in *traM* do not affect *traR* expression as monitored from a *traR*::*lacZ* fusion (data not shown). The apparent self-regulation of *traM* expression by TraM (Table 7) is most likely due to its suppressive effect on TraR-mediated gene activation, since the activator is required for *traM* expression (Table 7).

We favor a model in which TraM interacts directly with TraR. In our initial studies, inhibition of *tra* expression mediated by TraR and AAI was observed only when the *traR* clone was carried on the relatively low copy number (three to five copies per cell [9]) IncW vector pSa152 (Fig. 2A). Expressing *traR* from the high-copy-number (10 to 12 copies per cell [4]) IncQ vector pDSK519 alleviates the suppressive effect (Fig. 2A). However, suppression is reestablished if *traR* and *traM* are cloned in and expressed simultaneously from the same high-copy-number pDSK519 vector. This finding suggests that the relative levels of TraR to TraM are important for the suppression and is consistent with a model in which the two proteins interact with each other. This hypothesis also is supported by our observation that pHS10, which contains a large fragment of pTiC58 encoding both *traR* and *traM* in their native configurations, expresses the suppressive phenotype (Fig. 2B). Mutating *traM* on this construct abolishes the inhibition, consistent with our proposal that TraR resulting from basal-level expression of its gene (38) is now free to interact with AAI.

There is, at this time, no conclusive evidence that a TraM homolog exists in other autoinduction systems. However, Fuqua and Winans reported on the isolation of an insertion mutation in the wild-type octopine Ti plasmid pTiR10 that results in a *traM*-like Tra^c phenotype (22). The insertion is not located in the pTiR10 *traR* gene but rather maps between *traR* and *tra* in a region of this Ti plasmid known to be related to the *traAF-traR* region of pTiC58 (21). Moreover, autoinduction of the *phz* genes by the *luxR* homolog *phzR* in *Pseudomonas aureofaciens* is suppressed by a fragment of DNA cloned from a region downstream of the gene encoding the activator (37). It remains to be determined if this suppressive effect is due to a TraM homolog.

What then might be the purpose of TraM? *Agrobacterium* strains harboring wild-type pTiC58 are phenotypically conjugation negative in the absence of opine induction. However, they do produce very small amounts of AAI (15). Furthermore, our previous work suggests that *traR* can be expressed at very low levels from its own promoter, even in the absence of induction by the conjugal opines (38). Presumably, this basal level of expression provides the cells with priming amounts of TraR and AAI. In contrast, strains harboring *traM* mutants of pTiC58 are Tra^c and produce AAI at easily detectable levels. We propose that TraM acts to inhibit Ti plasmid transfer resulting from basal level expression of *traR* and *traI*. Zhang and Kerr reported that addition of exogenous AAI does not override the need for induction by opines (55). This finding is consistent with our observation that a large excess of exogenous AAI does not reverse the suppressive effect of TraM (Fig. 2B) and fits a model in which TraM interacts with TraR to sequester the activator from the available AAI. Furthermore, the relative expression of *traM* versus *traR* should be important. In the absence of the conjugal opines, sufficient amounts of TraM must be made to titrate the available TraR. Our finding that TraR activates expression of *traM* is consistent with this model and supplies a mechanism by which the cells can produce TraM protein at levels sufficient to inhibit the available TraR under conditions of basal-level expression. When the cells are exposed to the conjugal opine, the expression of *traR* is induced, and the resulting increased levels of TraR protein overcome the available TraM. This model is consistent with our finding that the suppressive effect of TraM can be overcome by increasing the copy number of the plasmid expressing *traR* but not by increasing the amount of AAI (Fig. 2). This observation also argues against an alternative mechanism in which TraM serves to bind and sequester AAI. If such were the case, supplying excess AAI should overcome the TraM effect, while overexpressing TraR should not. Following induction with the conjugal opine, free TraR is now available to interact with the small amounts of AAI already produced, and the putative TraR-AAI complex is activated to initiate transcription of the *tra* and *trb* operons. Expression of the latter, which encodes *traI*, results in increased production of AAI (25). This allows activation of more TraR protein, further potentiating the expression of the *tra* genes.

In this model, basal expression of TraR and AAI poises the cells for conjugal transfer. TraM, however, modulates the system such that transfer is not induced until the cells encounter the environment of a tumor producing the conjugal opines. Transconjugants receiving the Ti plasmid inherit the ability to catabolize the opines produced by the tumor. Thus, the system ensures that the energy-expensive process of conjugal transfer does not initiate until the conditions are favorable to the transfer process and also to the inherited capacity of the resulting transconjugants to utilize the opines.

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